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Fetuin-A: A Major Fetal Serum Protein that Promotes ''Wound Closure'' and Scarless Healing

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TO THE EDITOR

Burn-wound healing is a dynamic, interactive process involving a number of cellular and molecular events and is characterized by inflammation, granulation tissue formation, re-epithelialization, and tissue remodeling (Greenhalgh, 2002; Linares, 2002). Unlike incisional-wound healing, it also requires extensive re-epithelialization due to a predominant horizontal loss of tissue and often heals with abnormal

Abbreviation: 2D, two-dimensional

scarring when burns involve deep dermis. The early mammalian fetus has the remarkable ability to regenerate normal epidermis and dermis and to heal dermal incisional wounds with no signs of scarring. Extensive research has indicated that scarless healing appears to be intrinsic to fetal skin (McCallion and Ferguson, 1996; Ferguson and O'Kane, 2004). Previously, we reported a fetal burn model, in which 80-day-old ovine fetuses (gestation = 145–153 days) healed deep dermal partial thickness burns without scars, whereas postnatal lambs healed equal depth burns with significant scarring (Cuttle *et al.*, 2005; Fraser *et al.*, 2005). This burn model provided early evidence that fetal skin has the capacity to repair and restore dermal horizontal loss, not just vertical injuries.

THE PROTEOMIC PROFILING OF FETAL AND POSTNATAL LAMB SKIN

To search for potential molecules responsible for scarless wound healing of burns in the fetus, we initiated a proteomic investigation of our fetal ovine burn model. This fetal ovine burn experiment was approved by the University of Queensland Animal Ethics Committee. We used two-dimensional (2D)-PAGE) followed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. The 2D-PAGE patterns of protein expression were compared in four tissue samples: fetal control and burn and lamb control and burn (Cuttle *et al.*, 2005; Fraser *et al.*, 2005). The results show that the patterns of protein expression are similar between these four types of samples, characterized by two clusters of protein spots, one with p*I* 4–6 and medium sizes in molecular weight and another with p*I* 6–10 and very low molecular weight (Figure 1a–d). As predicted, there are differences, such as more protein spots with low-level expression in postnatal skin compared to fetal skin and the upregulation of a number of protein spots following burn injuries. Most noticeably, there are several proteins that appear to be expressed in a much higher level in fetal skin than in lamb skin.

Mass spectrometry analysis of 2D-PAGE spots has been widely employed to investigate the skin proteome (Huang *et al.*, 2003; Liang *et al.*, 2004; Hensbergen *et al.*, 2005). These studies profiled cultured keratinocytes and fibroblasts or epidermis only to examine their response to UV radiation or to identify the expression signatures of epidermal stem cells. The pattern of protein spots obtained from the *in vivo* skin sample of the current study is quite different from that seen in these earlier studies. Nevertheless, the 2D pattern of ovine postnatal skin sample does resemble that of murine skin with a number of high-level protein spots around p*I* 4–6 and medium size in molecular weight (Huang *et al.*, 2003). To our knowledge, this is the first report detailing the proteomic profiling of fetal skin.

HIGH LEVELS OF FETUIN-A IN OVINE FETAL SKIN

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry analysis identified fetuin-A as a major component of fetal skin (Figure 1a and b), and this was verified by western blotting using a fetuin-A antibody (a kind gift from Dr Dziegielewska). Fetuin-A, also known as alpha2-HS glycoprotein, is a product of the *AHSG* gene. A search of human keratinocyte and fibroblast databases (http://proteomics.cancer.dk) failed to reveal this protein. In the current study, fetuin-A protein was one of the most abundant proteins detected by silver staining and a high level of expression was maintained from fetus day 81 (F81) up to F140. After birth, fetuin-A expression was significantly reduced (Figure 1c and d) indicating that the high levels of fetuin-A are intrinsic to fetal skin. The quantitative analysis by western blot indicated an approximately fourfold increase in fetal control skin compared to lamb control skin, an approximately twofold upregulation following burn injuries for lamb skin, and no significant change following burn injuries for fetal skin (Figure 1e). Earlier work has shown that fetuin-A is mainly synthesized by hepatocytes and secreted into the peripheral blood circulation system with the highest concentrations found in the fetus (Pederson, 1944; Dziegielewska and Brown, 1995). A recent study detected fetuin-A in adult mouse skin by western blotting (Denecke *et al.*, 2003), but we found much higher levels in fetal ovine skin.

LOCALIZATION OF FETUIN-A IN OVINE SKIN

To examine the significance of fetuin-A expression in skin development and wound healing, we investigated the localization of fetuin-A in skin tissues. In agreement with the 2D-PAGE analysis, fetal skin expresses much higher levels of fetuin-A than postnatal skin (Figure 1f and n). Interestingly, in F81–94 day fetal skin, the highest level of fetuin-A expression was found in keratinocytes of the epidermal basal layer (Figure 1f), which also express high levels of keratin 14 (Figure 1i). Fetuin A is particularly prominent at sites where basal keratinocytes are reorganizing to form hair follicle placodes. However, from F101 day onward basal-layer expression of fetuin-A is lost, and by day F140 only low levels of fetuin-A are observed in the bulb region of hair follicles and outer root sheaths of hair follicles (data not shown). Fetuin-A is also found at high levels in the dermis during fetal development (F81–140 days) and particularly in fibroblasts under and adjacent to the developing placode (dermal condensate and fibrous sheath of hair follicle).

Unlike human and rodent hair follicles, the wool follicles of merino sheep consist of primary, secondary, and secondary-derived follicles, with the primary and secondary hair follicles developing at F70–90 days (Rogers, 2006). Thus, the onset of fetuin-A expression in the basal layer of the epidermis coincides with the initiation of primary and secondary hair follicle development. This may also be the case for other mammals. For example, in developing rat skin, fetuin-A is present in a subset of basal layer cells at embryo day 18 (E18) but not at E12–16 days (Terkelsen *et al.*, 1998). This expression at E18 skin corresponds to the appearance of hair germs (Gibson *et al.*, 1983). However, the patchy pattern of fetuin-A expression in the basal layer of rat skin is very different

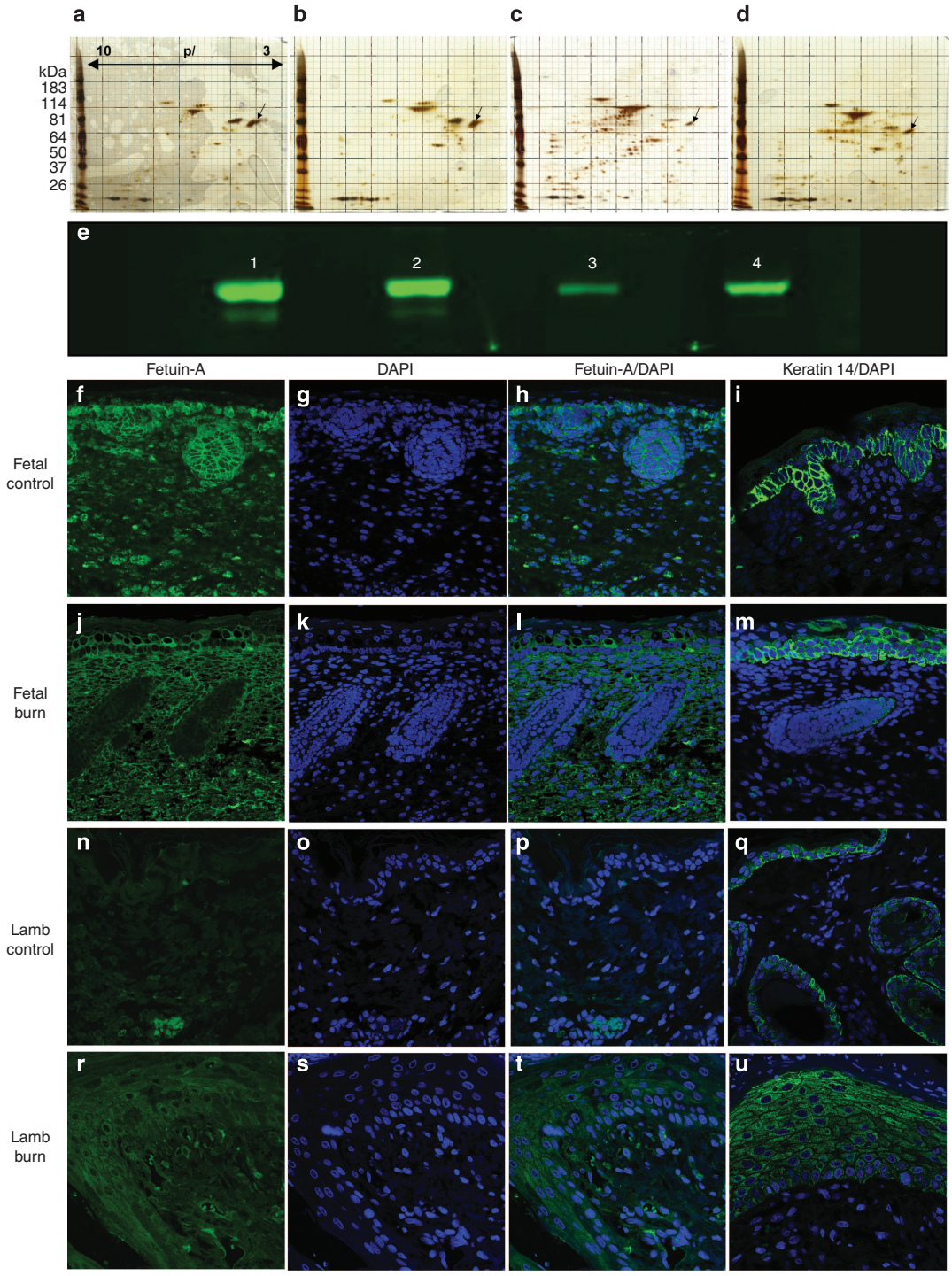

Figure 1. The expression of fetuin-A in skin samples. (**a**) The protein profiles of ovine fetal control skin at fetus 80 days, (**b**) ovine burned fetal skin day 1 post-burn injuries at fetus 81 days, (**c**) postnatal lamb control skin aged 30 days, (**d**) and burned postnatal lamb skin day 1 post-burn injuries aged 31 days. Samples containing 10 μg of skin protein were subjected to isoelectric focusing in linear gradient Immobiline Dry-Strips of pH 3–10, separated according to mass via SDS-PAGE, and then silver stained. Arrows indicate fetuin-A spots, which were identified by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry and further confirmed by western blotting. (**e**) Western blotting of same four skin samples from above (lane 1: fetal control, 2: fetal burn, 3: lamb control, 4: lamb burn). The total skin lysates were fractionated by SDS-PAGE and transferred to a polyvinylidine difluoride membrane. The blot was then probed with fetuin-A antibody (Acris, Acris Antibodies) and bound antibody was visualized by secondary antibody IRDye 800 (Rockland Immunochemicals Inc., Rockland, NY) on ODYSSEY (LI-COR Biosciences, Lincoln, NE). It shows that fetuin-A is more highly expressed in fetal skin than in postnatal lamb skin. (**f–i**) Sections (4 μm) of fetal control skin at fetus 81 days, (**j–m**) burned fetal skin 7 days post-burn injuries, (**n–q**) postnatal lamb control skin aged 37 days, and (**r–u**) burned postnatal lamb skin 7 days post-burn injuries aged 37 days were treated with antibodies to (**f**, **j**, **n**, **r**) fetuin-A or (**i**, **m**, **q**, **u**) keratin 14. (**f**) Bar = 10 μm. The tissue binding of these primary antibodies are visualized with Alexa Fluor 488. 4′,6-Diamidino-2-phenylindole was used for DNA staining. Fetuin-A is highly expressed in (**f**) the basal layer of epidermis in fetal skin, (**j**, **r**) and in the suprabasal layer of epidermis post-burn injuries.

from the uniform expression seen in basal-layer keratinocytes in the current study. This could be explained by a number of differences in skin morphology and skin appendages between these two species. It is well known that the inhibition of bone morphogenetic protein signaling in dermis and the activation of Wnt signaling in epidermis act in concert to induce and initiate hair follicles (Millar, 2002; Fuchs, 2007). Fetuin-A is a natural antagonist of bone morphogenetic protein (Demetriou *et al.*, 1996), and the high levels of fetuin-A in the dermis, particularly surrounding developing hair placodes and hair germs, may be indicative of a role in regulating follicle growth and/or initiation by modulating bone morphogenetic protein signaling.

FETUIN-A EXPRESSION IN BURNED SKIN TISSUE

Although the expression level of fetuin-A is not significantly changed following burn injuries, its expression is clearly upregulated in suprabasal layers of the epidermis in both burned fetal (Figure 1j) and postnatal lamb skin (Figure 1r), whereas fetuin-A is undetectable in the suprabasal layer of normal control skin. This upregulation is maintained for about 2 weeks post-burn injury. At the same time, keratin 14 expression is seen in both basal and suprabasal layers of epidermis (Figure 1m and u), which has been suggested as being essential for the epidermal integrity of healing wounds (Patel *et al.*, 2006). Nevertheless, dermal fetuin-A expression after burn injury remains at the same level and pattern. It is unclear what role the expression of fetuin-A in the suprabasal cells plays in this process. As one of the mechanisms for wound healing is the re-activation of developmental processes (Stramer and Martin, 2005), the appearance of fetuin-A in these cells may reflect a role in tissue reorganization.

FETUIN-A PROMOTES WOUND CLOSURE IN PRIMARY KERATINOCYTES

We speculate that fetuin-A may be involved in re-epithelialization. To test this hypothesis, we used a well-described and widely used *in vitro* wound-closure assay (Li *et al.*, 2004; Greenhalgh, 2005). Human foreskin primary keratinocytes were cultured to confluence on 24-well plates and a cross-like wound (≈1 mm wide) was created by ''scraping'' (Figure 2a). The cultures were then incubated in keratinocyte-serum-free media (Invitrogen, Carlsbad, CA) with or without fetuin-A. Wound closure was monitored and quantified by image analysis (Image Pro Plus) at 24 hours post-wounding. The results showed a reduction in wound size in wells containing fetuin-A (Figure 2). In cultures with 500 μg ml^{-1} fetuin-A, wound closure was most significant with nearly 80% of the wound closed, compared to only 30% closure in cultures without fetuin-A. Interestingly, enhanced wound closure by fetuin A was due mostly to increased cell migration rather than to increased levels of proliferation, as judged by bromodeoxyurine staining of cultures with fetuin A (data not shown).

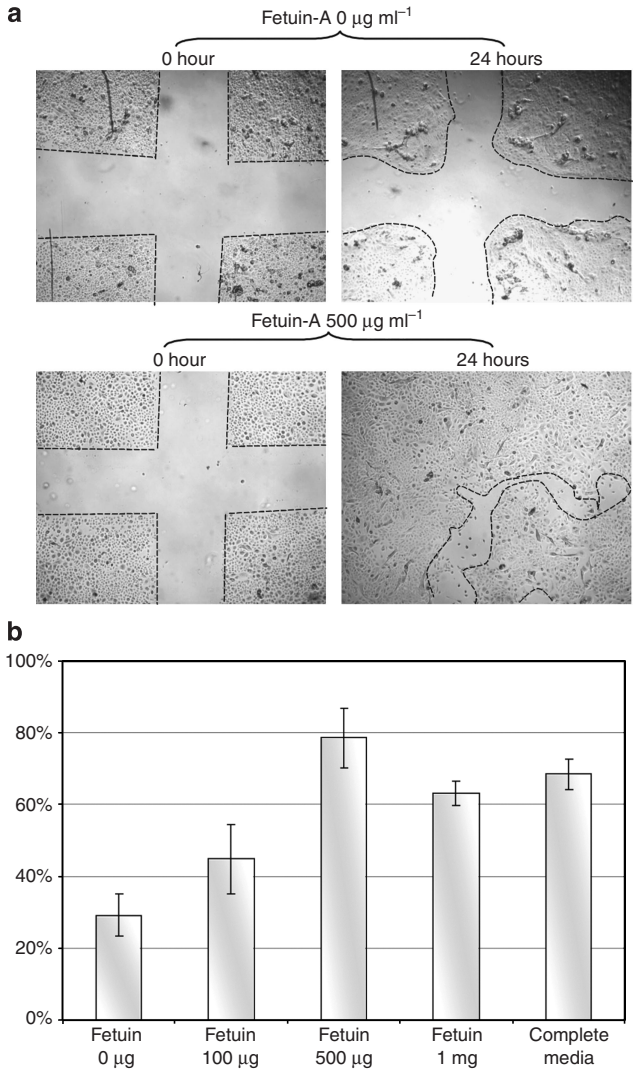

Figure 2. Fetuin-A promotes wound closure on human primary keratinocyte culture. Human foreskin primary keratinocytes were plated in 24-well plates and allowed to proliferate to full confluence. A cross-like wound was created and cultures were then incubated with or without fetuin-A 100 μg–10 mg ml^{-1} in keratinocyte-serum-free media. Keratinocyte-serum-free media supplemented with EGF and bovine pituitary extract is referred to as keratinocyte complete media and is used as a positive control. At 24 hours post-wounding the wound closure was monitored and quantified by an image-analysis program. (**a**) Two examples of a wound-closure assay. The dotted line indicates the areas of the wound at 0 and 24 hours. (**b**) The quantitative analysis of keratinocyte wound closure with different concentrations of fetuin-A. The percentage represents the area of wound closed compared with the original wound size.

Wound re-epithelialization and keratinocyte migration are initiated by exposure to various growth factors and cytokines released within wounds by damage tissue. Earlier closure of the wound is a desired goal in burn-wound healing in order to minimize scarring. The findings from the current study show that fetuin-A is able to markedly enhance wound closure in primary keratinocytes, and is present as a major protein component of fetal skin. It shows regional localization during skin development and wound healing, suggesting that fetuin-A plays an important role in these processes. Moreover, fetuin A has already been implicated in a number of growth factors/cytokine pathways, which are involved in inflammatory response, granulation tissue formation, and tissue remodeling of wound healing. The current findings provide direct supporting evidence that fetuin A may contribute to scarless wound healing.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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